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Higher Fatty Acid Derivatives of Proteins

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A series of novel fatty acid derivatives of proteins, prepared by the reaction of acid chlorides with proteins dissolved in aqueous alkali, is described. The influence of a number of experimental variables on the extent of acylation was investigated. The preparative procedure adopted readily gives derivatives of casein which are acylated to the extent of approximately 20% by substituent groups ranging from caprylyl to stearoyl. This procedure was also used for the preparation of palmitoyl derivatives of egg albumen, zein, wheat gluten, and soybean, peanut, and cottonseed proteins. Physical and chemical properties of a representative derivative, palmitoyl casein, are discussed. Among noteworthy characteristics of the acylated products are their reduced affinity for water and their altered solubilities.

ACYLATION of casein by acetic, propionic, and butyric anhydrides was described in a recent paper from this laboratory (*13*). Evaluation of the acylated products as plastic molding powders led to the conclusion that they were superior to casein in so far as a materially curtailed water absorption was concerned but inferior with respect to brittleness and strength. The results suggested that acylation of casein by higher fatty acid residues might possibly yield derivatives in which the former desirable property would be retained or enhanced without sacrifice of the toughness and strength characteristic of casein plastics. The authors attempted to achieve these ends by modifying casein and other proteins with longer chain aliphatic acid chlorides in aqueous alkaline solutions as well as in the presence of pyridine. The preparation and properties of these modified proteins, of some interest in themselves as representatives of a rather unusual type of protein derivative, are described here.

A survey of the literature revealed a few papers dealing with the preparation of higher fatty acid derivatives of amino acids and proteins. Bondi and co-workers (*4, 5, 6*), Abderhalden and Funk (*1*), Izar (*15*), and Koebner (*17*) obtained acylated products by the action of various higher fatty acid chlorides on amino acids, peptides, and peptone in aqueous alkaline solutions, according to the Schotten-Baumann technique. Similar derivatives of degraded proteins are mentioned in a British patent (*9*). Izar and DiZuattro (*16*), also using this technique, prepared a large number of similarly modified proteins, but the individual products were not described in detail, so that little of importance to our work could be learned from this article; in fact, it would appear from the data presented that the derivatives were quite different from those to be described here. We were able to find only one publication, a French patent (*20*), which deals with acylated proteins apparently comparable to ours. The patent reports the modification of casein by fatty acid chlorides in pyridine, but again characterization of the products is lacking.

PREPARATION OF PALMITOYL CASEIN

The detailed procedure finally adopted for the preparation of palmitoyl casein will be described before the effects of experimental conditions are considered. Twenty grams of casein are stirred mechanically with 180 ml. of water, and, after 15 minutes, 20 ml. of *N* sodium hydroxide are added. When the casein is dissolved (about 0.5 hour), a suitable glass electrode (Beckman Type E gh pH) is immersed in the solution. Meanwhile, a solution of .3 ml. of palmitoyl chloride in 50 ml. of dry ether is prepared in a separatory funnel. The pH of the casein solution is adjusted to 12 by the dropwise addition of 2 *N* sodium hydroxide. The acid chloride solution is then run in at such a rate that it is all added in 7-10 minutes, and, simultaneously, 2 *N* sodium hydroxide is added to maintain the pH at 12. Proper control of pH is largely dependent upon efficient mixing. About 5 minutes after all the acid chloride has been added, the mixture thickens appreciably; at the end of another 15 minutes, consumption of alkali becomes very slow. Vigorous stirring is continued, and the addition of 50 ml. of water may be needed to facilitate mixing. At the end of a 1-hour reaction period, timed from the start of the addition of acid chloride, the viscous mixture is poured into 1 liter of water. With vigorous stirring, 6 *N* hydrochloric acid is added dropwise to pH 4.0 to precipitate the derivative. The mixture may be heated to 70° C. on the steam bath at this point to drive off the ether, since its presence, especially in large batch preparations, causes subsequent filtration to be slow. If the mixture has been heated, it is again cooled to room temperature and then filtered. The filter cake is washed with water until the filtrate is free of chloride ion, and the greasy cake is broken up and allowed to dry in air. The air-dried material is ground and Soxhlet-extracted with ether for at least twenty-four hours. The yield of final ether-insoluble product, a white powder, is 23.9 grams or 121% of the dry weight of the original casein.

ANALYTICAL METHODS. The modified protein samples were extracted with ether in the preparative procedure to remove gross free fatty acid. They were then dried in the air before analysis. Moisture and Kjeldahl nitrogen determinations were carried out according to the recommendations of Chibnall *et al.* (*10*). Moisture contents were determined simultaneously with the analyses for other constituents, and all analytical figures were calculated on a moisture-free basis. Total ash was estimated essentially according to the calcium acetate method described by Sutermeister and Browne (*21*). Since all modified protein samples during the course of their preparation were washed with water until free of chloride ion, their ash contents were not greatly different (2.3 ± 1.0%) and will not be recorded individually. Amino nitrogen was determined by the Van Slyke method as employed by Doherty and Ogg (*11*).

In spite of the fact that all derivatives were extracted with ether during the course of their preparation, the complete removal of free fatty acid was rather difficult (*19*). The routinely extracted samples were, therefore, analyzed for residual free fatty acid as follows: Duplicate 2-gram samples, in sintered glass extraction thimbles, were extracted with ether in the Soxhlet apparatus to constant weight. When constant weight was attained, it was assumed that free fatty acid was no longer present. It was found by this method that about 1 to 2% free fatty acid was usually present in the routinely extracted samples, and an appropriate correction was applied to the analytical figures. The chemically bound fatty acid content of the products was also determined. Various methods of hydrolysis were employed in attempting to remove the acyl groups completely. Hydrolysis with alkali under relatively mild conditions, which suffice to cleave acetyl groups quantitatively from protein derivatives (*14*), was

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TABLE I. COMPARISON OF COMBINED FATTY ACID ISOLATED BY HYDROLYSIS WITH THAT CALCULATED FROM DECREASE IN NITROGEN CONTENT OF CASEIN DERIVATIVES

Expt. No.	Sample No.	Acid Substituted	N Content[a], %	Acyl Content Calcd.[b] %	Acyl Content Found[c], %	Recovery[d], %
1	38-8	Palmitic	13.34	10.2	10.3	101.0
2	31-142	Palmitic	11.74	21.0	20.5	97.6
3	31-145	Palmitic	11.38	23.4	23.0	98.3
4	31-150	Palmitic	12.11	18.5	18.0	97.3
5	31-137	Oleic	11.32	23.8	23.2	97.5
6	31-162	Oleic	11.45	22.9	22.3	97.4
7	31-181	Oleic	12.63	15.0	13.9	92.7

[a] Nitrogen content of unmodified casein, 14.86%.
[b] Calculated from decrease in nitrogen content of derivative.
[c] Calculated from fatty acid isolated after hydrolysis.
[d] Acyl content from decrease in nitrogen content taken as 100%.

TABLE II. ACYLATION OF CASEIN BY PALMITOYL CHLORIDE UNDER VARIOUS EXPERIMENTAL CONDITIONS

Expt. No.	Sample No.	Acid Chloride Concn., Moles/100 G. Casein[a]	Time, Hr.	pH	N Content[b], %	Acyl Content, %
1	38-12[c]	...	2	12	14.67	...
1A	31-132[d]	...	2	12	15.00	...
2	31-118	0.2	2	12	13.83	6.9
3	38-10	0.4	2	12	11.94	19.6
4	38-6	0.6	2	12	11.35	23.6
5	38-61	0.4	1	12	11.52	22.5
6	31-125	0.4	5	12	11.71	21.2
7	31-145	0.4	2	11	11.38	23.4
8	31-142	0.4	2	10	11.74	21.0
9	31-187	0.4	2	9	12.18	18.0
10	38-30	0.4	2	8	12.79	13.9

[a] Concentration of casein was 10 grams per 100 ml. alkali at the start of the reaction.
[b] Nitrogen content of original casein, 14.86%.
[c] Control: all conditions identical with those of experiment 3, except that 0.4 mole of palmitic acid per 100 grams of casein was added instead of acid chloride.
[d] Control: conditions same as experiment 3, except that 0.6 mole of palmitic acid per 100 grams of casein was added.

ineffective in liberating palmitoyl groups completely from palmitoyl casein. A similar difficulty in removing benzoyl groups from benzoylated proteins has been reported (*12*). Hydrolysis with 25% sodium hydroxide for 24 hours at reflux temperatures did yield maximal values for combined fatty acid, but difficulties with foaming and precipitation of silicates upon acidification of the hydrolyzate led to abandonment of further study of alkaline methods. Bondi and Eissler (*5*) used acid hydrolysis to remove lauroyl groups from a peptone derivative. Accordingly, hydrolytic procedures with acid were investigated, and, as conditions were made more drastic, the yield of fatty acid isolated increased until it was possible to obtain maximal values. These values were in close agreement with figures for acyl content calculated from the differences in the nitrogen contents of the unmodified and the acylated proteins. A summary of the method finally employed follows.

The residue from the free fatty acid determination is used. To 1-gram samples, 50 ml. of sulfuric acid (1 volume of concentrated acid added to 3 volumes of water) are added. The mixtures are heated on the steam bath for about 30 minutes and then boiled gently under reflux for 24 hours. Each hydrolyzate, after cooling, is extracted with five 40-ml. portions of ether in a separatory funnel, and the combined ether extracts are washed with three 75-ml. portions of water. The ether extract is then evaporated to dryness in a tared dish on the steam bath. Final drying to remove small amounts of water is accomplished in a vacuum desiccator over concentrated sulfuric acid, and the fatty acid residue is then weighed. Blank determinations on approximately equivalent amounts of ether-extracted unmodified protein give values of about 1.0%, and this correction is applied.

Recovery experiments in which known amounts of palmitic acid were added to unmodified casein before hydrolysis gave yields of about 99% of the acid added. However, the yields of fatty acid after hydrolysis of the acylated caseins were usually a little low, ranging in most cases from 95 to 98% of the amounts calculated to be present from nitrogen determinations; in a few instances, yields of only 93% were obtained. Since no absolute check of the method is possible with protein compounds of known composition, specific claims as to its accuracy can be made. Representative data on fatty acid isolated, as compared with that estimated to be present from nitrogen analyses, are summarized in Table I. Equally close agreement between similar pairs of values was obtained with lauroyl, myristoyl, and stearoyl caseins. It is believed, therefore, that the changes in nitrogen content occasioned in the modification experiments constitute a reliable index of the extent of acylation; in subsequent tables figures are presented calculated on this basis alone.

EFFECT OF CONDITIONS. Exploratory experiments were carried out on the acylation of casein by palmitoyl chloride, both in aqueous media and in pyridine. Since the former technique was preferable for a number of reasons, it was employed in most of the work. The disadvantages of modification in pyridine will be pointed out in a subsequent section. The initial experiments in aqueous alkali, according to the Schotten-Baumann technique as applied to amino acids, produced palmitoyl casein in excellent yield in the form of a white powder. However, it was extremely difficult to obtain uniform products, presumably because of the diversity of functional groups in the protein molecule. Accordingly, a detailed study of experimental conditions was initiated, and the following variables were investigated: purity of acid chloride, concentration of casein in the reaction medium, moles of acid chloride per unit weight of casein, reaction time and temperature, and pH of the reaction medium. The casein used throughout this series of experiments was a commercial hydrochloric acid–precipitated product. Sodium hydroxide was employed as the alkali.

The purity of the palmitoyl chloride, within reasonable limits, had little effect on the course of the reaction. This and other fatty acid chlorides were prepared by the action of phosphorus trichloride on commercial acids of the Armour Neo-fat type in the manner of Aschan (*2*). After the phosphorous acid sludge was drawn off, the light yellow product was heated on the steam bath in vacuo to remove small amounts of excess phosphorus trichloride, although this step was probably unnecessary. Pure distilled palmitoyl chloride and Armour's commercial product yielded modified proteins not significantly different from those made with our acid chloride.

The concentration of casein in the reaction mixture is limited by physical considerations. Ten grams of casein in 100 ml. of aqueous alkali at the start of the reaction is approximately the upper limit of concentration, because the mixture becomes extremely viscous during the course of the reaction, and efficient stirring and control of pH become progressively more difficult. A few experiments in which 3 and 5% solutions of casein were used gave less satisfactory modification. A likely explanation for this finding cannot be offered, but since it was desired to employ as concentrated a solution of the protein as possible, all further work was done with 10% solutions, and the point was not studied further.

Reactions were conducted at 5° C. and at room temperatures. When acid chlorides above C_{12} in chain length were used, the different temperatures had little effect on the course of the reaction; therefore subsequent experiments were performed at room temperature. However, reactions with acetyl, butyryl, and caproyl chlorides were run in an ice bath to minimize hydrolysis of the chloride.

Effects of the remaining variables investigated are summarized in Table II. Experiments 2, 3, and 4 illustrate the effect of varying the ratio of acid chloride to casein. The lowest ratio tried, 0.2 mole of acid chloride per 100 grams of casein, provides approximately three times the amount of chloride needed to react with the free amino groups of this protein. Although the most

TABLE III. CASEIN DERIVATIVES MADE WITH VARIOUS FATTY ACID CHLORIDES[a]

Sample No.	Acid Chloride Used[b]	N Content, %	Acyl Content, %	Acyl Content Moles/100 G. Casein
38-74[c]	Acetyl	14.56	2.0	0.05
38-80[c]	Butyryl	14.39	3.2	0.05
38-85[c]	Caproyl	13.80	7.1	0.08
38-93	Caprylyl	11.67	21.5	0.22
38-87	Pelargonyl	12.32	17.1	0.15
38-98	Capryl	11.75	20.9	0.17
38-64	Lauroyl	11.33	23.7	0.17
38-76	Myristoyl	10.99	26.0	0.17
38-61	Palmitoyl	11.52	22.5	0.12
31-137	Oleoyl	11.32	23.8	0.12
31-152	Stearoyl	11.41	23.2	0.11

[a] Reactions were carried out at room temperature, at pH 12, and with 0.4 mole of acid chloride per 100 grams of casein, unless otherwise noted.
[b] Radical names used throughout this paper are those recommended by *Chemical Abstracts* as listed in the 1943 index, and are not necessarily those used in the original references cited.
[c] Prepared at 5° C., pH 9 to minimize hydrolysis, and with 0.6 mole of acid chloride per 100 grams of casein; reactions were also run in this manner at pH 12 but more highly acylated products were not obtained.

highly substituted products were obtained when 0.6 mole of acid chloride per 100 grams of casein was employed, the effect was not consistent. For this reason, and also to reduce the substantial amounts of fatty acid formed by hydrolysis, 0.4 mole was used subsequently.

The reaction was not affected greatly by varying the time from 1 to 5 hours (experiments 3, 5, and 6). In general, the consumption of alkali required to maintain the pH is a good index of the course of modification. When this slows up, as it does soon after the addition of acid chloride is complete, the reaction is essentially at an end. However, we preferred to continue mechanical stirring and very slow addition of alkali as needed for some time thereafter. Usually a reaction period of 1 hour was used for small batches of casein (20 grams), and two hours for larger batches (300–600 grams).

Experiments 3, 7, 8, 9, and 10 appear to indicate that in the pH range 8 to 11 increasing alkalinity promotes substitution but that at pH 12 substitution falls off. The results of many other experiments make it necessary to modify this conclusion. It can be stated with some degree of assurance that substitution does increase progressively within the pH range 8 to 10. At higher pH values the results are inconsistent. The acyl content of preparations at pH values of 11 and 12 is usually higher, but occasionally lower, than that of samples made at pH 10, and substitution at pH 11 is often higher than that at 12. Accurate control of pH at these high levels and with viscous mixtures is manifestly difficult, and hydrolytic cleavage in this pH range must also be considered as a possibility. Since we were interested in exploiting the modification reaction to the fullest extent, the inconsistency of results within the pH range 10 to 12 made it difficult to decide on a single pH value which would lead to maximal substitution in comparative experiments with other acid chlorides and with other proteins. Because the most highly substituted products were most often obtained at pH 12, this pH was chosen for the comparative experiments.

Experiments 1 and 1A in Table II are control experiments carried out at pH 12 in exactly the same way as the modification experiments, except that palmitic acid was used instead of acid chloride. The results confirm our assumption that a decreased nitrogen content is a reliable index of modification and demonstrate, further, that the procedures employed for purification of the derivatives completely remove fatty acid not substituted in the protein molecule.

OTHER ACYLATED CASEINS

When the study of experimental conditions for the preparation of palmitoyl casein was completed, the reaction was applied to the protein with other fatty acid chlorides to allow observing the effect of chain length of the substituent group on the course of the reaction and on the nature of the products. The experiments with acid chlorides ranging from acetyl to palmitoyl chlorides are summarized in Table III. Fewer functional groups in casein were acylated by the shortest and longest chain acid chlorides used, but the acyl content in per cent did not vary greatly with acid chlorides ranging from C_8 to C_{18}. The products within this range did not appear to differ materially in their properties.

Casein was also treated with succinyl, adipyl, and sebacyl chlorides by the same technique employed with the monobasic acid chlorides. Decreases in the nitrogen contents of the derivatives equivalent to 10–12% substitution were obtained. However, the products appeared to be inferior as potential plastic molding powders and were not studied further.

PALMITOYL DERIVATIVES OF OTHER PROTEINS

Acylation of proteins other than casein with palmitoyl chloride was accomplished by the same technique described for the preparation of palmitoyl casein at pH 12. The reaction seems to be applicable generally to proteins which can be dissolved in aqueous alkali. Its application to proteins insoluble in alkali was not investigated. Table IV shows the extent of acylation obtained with a number of proteins of industrial significance. All the proteins except egg albumen and gluten gave products in amounts at least equal to the original weights of protein used; palmitoyl albumen and gluten were obtained in yields of about 90% on this basis. The palmitoyl zein is of particular interest because of the low amino nitrogen content of this protein. Complete acylation of the phenolic hydroxyl groups in zein would account for a fraction of the substituent groups, but the greater portion must be attached elsewhere. Control experiments demonstrated that the nitrogen content of zein is not altered appreciably by treatment with alkali as in the modification procedure; so acylation of the numerous amide groups of this protein is a possibility. However, if experiments with simple amides may be considered as models (*22*), it must be admitted that such acylation is improbable.

The solubilities of the modified proteins are quite different from those of the original proteins. Palmitoyl egg albumen is no longer soluble in water and palmitoyl zein is only slightly soluble in cold 70% ethanol. However, palmitoyl zein is soluble in a number of chlorohydrins and chloroallyl alcohols, and the clear solutions may be used to cast films.

CHARACTERIZATION OF PALMITOYL CASEIN

SOLUBILITY. Palmitoyl casein is insoluble in water, dilute acids, and the common organic solvents tested, but it is soluble in ethylene chlorohydrin and β-chloroallyl alcohol. It yields a turbid suspension in cold dilute alkali, which is transformed into a clear, colorless solution on warming.

TABLE IV. PALMITOYL DERIVATIVES OF VARIOUS PROTEINS

Sample No.	Protein and Source	N Content of Unmodified Protein[a], %	N Content of Derivatives, %	Palmitoyl Content[b], %
31-173	Egg albumen (sol., Eimer and Amend)	13.47	11.16	17.1
38-65	Zein (Corn Products Refining Co.)	15.44	11.90	22.9
38-51	Soybean (alpha, Glidden)	14.78	11.90	19.5
38-53	Soybean (ortho, Drackett)	14.72	11.68	20.7
38-55	Peanut[c]	16.41	12.85	21.7
38-57	Cottonseed[c]	16.34	12.73	22.1
38-59	Wheat gluten (Pfanstiehl)	14.26	12.67	11.2

[a] Figures listed in this column are for the proteins as received and are corrected only for moisture; purification of the unmodified proteins was not attempted.
[b] Calculated from differences in nitrogen content. With the exception of the figure for palmitoyl zein, which was checked by direct analysis for combined palmitic acid, these values must be considered as rough estimates (see footnote [a]).
[c] Furnished by the Oil, Fat, and Protein Division of the Southern Regional Research Laboratory.

DENSITY. The approximate densities of casein derivatives of varying palmitoyl content were determined by observing whether they floated or sank in bromobenzene-xylene mixtures of known specific gravities (*3*). The values obtained for the derivatives were significantly lower than that for unmodified casein. For example, a density of 1.18 was found for palmitoyl casein containing 22% acyl groups, whereas that for unmodified casein was 1.27.

BEHAVIOR IN DILUTE ALKALI. Derivatives with palmitoyl contents greater than 15% had amino nitrogen contents of 0.05 to 0.12%. These values indicate clearly that most of the amino groups in the original casein (0.84% amino nitrogen) were acylated. The behavior of acylated caseins on treatment with dilute sodium hydroxide often provides information concerning the distribution of acyl groups because of the lability of O-acyl groups and the stability of N-acyl groups in this medium. Partition experiments of this type have been carried out with acetylated casein (*7*, *13*, *14*). In similar experiments two samples of palmitoyl casein were treated with 0.1 *N* sodium hydroxide at room temperature for 6 hours, conditions which suffice to remove O-acetyl groups from acetylated casein. The first sample, containing 20.4% palmitoyl, yielded a product of 18.7% palmitoyl content; corresponding figures for the second sample were 18.8 and 17.2% palmitoyl. When the treatment with alkali was extended to 24 hours, the palmitoyl contents of the two samples were 16.9 and 16.2%, respectively. Since complete substitution of the free amino groups in casein requires theoretically only about 13% palmitoyl, it seems likely that most, if not all, of the amino groups in these derivatives were acylated. This is in agreement with the low amino nitrogen values cited for similar derivatives. It is also apparent from the partition experiments that palmitoyl casein is considerably more stable to dilute alkali than is acetyl casein. Incidentally, a good part of the palmitoyl groups in palmitoyl zein is stable in dilute alkali, although zein contains few free amino groups.

BEHAVIOR TOWARDS MILLON'S REAGENT. Palmitoyl casein containing 20% palmitoyl gave a negative Millon's test. After this derivative was treated with dilute alkali for 24 hours as described in the preceding paragraph, the product gave a strongly positive test. Presumably O-palmitoyl groups had been hydrolyzed from substituted tyrosine residues. A palmitoyl casein containing only 10.3% acyl group also gave a positive Millon's test.

REACTION WITH FORMALDEHYDE. When palmitoyl casein (15 to 20% palmitoyl groups) was treated with formaldehyde, the resulting product contained approximately 1.5% formaldehyde, only about half the amount bound by unmodified casein under the same conditions. The methods used for treatment with formaldehyde and analysis for bound aldehyde were identical with those previously employed for acetyl casein (*13*). The reduced formaldehyde-binding capacity observed for acetyl casein is thus also characteristic of palmitoyl casein. [Nitschmann and Lauener (*18*) recently reported that casein acetylated with ketene and then hardened with gaseous formaldehyde takes up almost as much formaldehyde as unacetylated casein. This is in marked contrast to the reduced formaldehyde-binding capacity of our preparations, which were acetylated with acetic anhydride and then treated with formaldehyde solution (*13*).] Acylation of groups otherwise free to react with aldehyde undoubtedly accounts for these observations, which are in accord with the results of Carpenter on acylated amino acids (*8*).

PYRIDINE AS A REACTION MEDIUM

Since acylation of protein by higher fatty acid chlorides in the presence of pyridine has been described (*20*), several samples of palmitoyl casein were prepared by this method for comparative purposes. Twenty-five grams of finely pulverized, anhydrous casein were stirred vigorously with 62 grams of anhydrous pyridine at 50–60° C., and 50 grams of palmitoyl chloride were added dropwise. The reaction mixture became dark brown in one hour. The reaction was run for 26 hours, and then the mixture was poured into warm ethanol. The colored precipitate was filtered, washed with ethanol, and then extracted with ether. A reddish-brown powder (30.5 grams) was obtained. When the product was washed with water, some water-soluble material was removed. The products isolated from a number of similar reactions with palmitoyl and stearoyl chlorides at temperatures ranging from 50° to 90° C. were all highly colored in contrast to the white or yellowish products obtained by the Schotten-Baumann technique. The anhydrous conditions and the cost of pyridine are also disadvantages of this procedure. Finally, although some of these preparations had low nitrogen contents, indicative of extensive substitution, they were inferior as potential plastic molding powders.

CONCLUSIONS

The affinity of casein and a number of other industrial proteins for water may be reduced by acylation in aqueous alkali with higher fatty acid chlorides. Under otherwise identical conditions maximal acylation of casein is favored at the higher pH values investigated—namely, pH 10 to 12. Fatty acid chlorides ranging from C_8 to C_{18} in chain length yield essentially similar casein derivatives. The products, which may contain as much as 26% fatty acid radical, are obtained in amounts usually larger than the original weights of unmodified protein. Substitution occurs at the free amino and phenolic hydroxyl groups of the protein and probably at other reactive sites.

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